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In vitro effect of nicorandil on the carbachol-induced contraction of the lower esophageal sphincter of the rat



Tomonori Shimbo, Takeshi Adachi, Susumu Fujisawa, Mai Hongoh, Takayoshi Ohba, Kyoichi Ono*

Department of Cell Physiology, Akita University Graduate School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan

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ABSTRACT

The lower esophageal sphincter (LES) is a specialized region of the esophageal smooth muscle that allows the passage of a swallowed bolus into the stomach. Nitric oxide (NO) plays a major role in LES relaxation. Nicorandil possesses dual properties of a NO donor and an ATP-sensitive potassium channel (K_{ATP} channel) agonist, and is expected to reduce LES tone. This study investigated the mechanisms underlying the effects of nicorandil on the LES. Rat LES tissues were placed in an organ bath, and activities were recorded using an isometric force transducer. Carbachol-induced LES contraction was significantly inhibited by K_{ATP} channel agonists in a concentration-dependent manner; pinacidil >> nicorandil ≈ diazoxide. Nicorandil-induced relaxation of the LES was prevented by pretreatment with glibenclamide, whereas N^G-nitro-L-arginine methyl ester (L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and iberiotoxin were ineffective at preventing nicorandil-induced LES relaxation. Furthermore, nicorandil did not affect high K⁺-induced LES contraction. Reverse-transcription polymerase chain reaction analysis and immunohistochemistry revealed expression of *KCNJ8* (Kir6.1), *KCNJ11* (Kir6.2), *ABCC8* (SUR1) and *ABCC9* (SUR2) subunits of the K_{ATP} channel in the rat lower esophagus. These findings indicate that nicorandil causes LES relaxation chiefly by activating the K_{ATP} channel, and that it may provide an additional pharmacological tool for the treatment of spastic esophageal motility disorders.

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1. Introduction

The lower esophageal sphincter (LES) is a specialized region of the esophageal circular smooth muscle at the junction between the esophagus and the stomach. It allows the passage of a swallowed bolus into the stomach while preventing the reflux of gastric contents into the esophagus (1–4). The LES is composed of at least two separate muscle components, the circular and the oblique sling muscles. Two muscles are functionally different; the sling muscle has little intrinsic myogenic tone but contracts vigorously to cholinergic stimulation, and the circular muscle is characterized by a spontaneously generated basal tone that helps to prevent reflux of gastric contents into the esophagus. The basal tone of the LES is primarily myogenic and can be modulated by enteric motor neurons, the parasympathetic and sympathetic extrinsic nervous system and

several neurohumoral substances (3). Dysfunction of LES motility is an important factor in the pathogenesis of gastroesophageal reflux disease, esophageal dysmotility and esophageal hypersensitivity (5–7). Such disorders often cause recurrent, angina-like, retrosternal chest pain. After excluding a cardiac cause, they are treated with medical, endoscopic and surgical therapeutics (6).

Nitric oxide (NO), synthesized by neuronal nitric oxide synthase (NOS), is the main inhibitory neurotransmitter involved in LES relaxation (1,3,8,9). NO is also synthesized in smooth muscles cells by a spontaneously active constitutive NOS, called myogenic NOS, and contributes to the resting membrane potential and tone (10,11). It is generally accepted that NO activates the large conductance K⁺ channel (BK_{Ca}) and causes membrane hyperpolarization. This mechanism potentially leads to LES relaxation (11–15). Exogenous NO is also able to cause LES relaxation, and therefore, spastic motility disorders of the esophagus can be treated with nitrate as well as Ca²⁺ channel blockers and phosphodiesterase type 5 inhibitors (6).

Nicorandil possesses dual properties as an NO donor and an ATP-sensitive potassium channel (K_{ATP} channel) agonist, and is

* Corresponding author.

E-mail address: onok@med.akita-u.ac.jp (K. Ono).

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widely used as an anti-angina drug. K_{ATP} channels composed of inward-rectifying potassium channel subunits (Kir6.1 and Kir6.2, encoded by *KCNJ8* and *KCNJ11*, respectively) and regulatory sulfonylurea receptor (SUR1 and SUR2, encoded by *ABCC8* and *ABCC9*, respectively). Alternative RNA splicing can give rise to multiple SUR protein variants (e.g., SUR2A and SUR2B) that confer distinct physiological and pharmacological properties on the channel complex (16–22). The drug activates K_{ATP} channels and relaxes not only vascular smooth muscles but also smooth muscles of other tissues, including those of the uterus, airway, anal sphincter, stomach, and ureter (16–22). It is therefore possible that nicorandil reduces LES contraction by dual mechanisms. The aim of this study was to elucidate the mechanisms underlying the effects of nicorandil on LES tone in the rat.

2. Materials and methods

2.1. Measurement of LES tension

All animal experiments were approved by The Animal Ethics Committee of Akita University School of Medicine (No. a-1-2601). Fifty eight male Wister rats weighing 230–520 g were used in this study. After inducing anesthesia with intraperitoneal injection of thiopental sodium (50 mg/kg), an incision was made to open up the abdominal and thoracic cavities, and the esophagus including the LES was excised. Excess fat and connective tissue were dissected and the LES was set up as a ring segment of 3 mm in width. The LES ring preparation was placed in a standard organ bath filled with normal Tyrode's solution. The LES ring was tied to a stainless steel hook at one end of the organ bath, and the other end was connected to a force transducer. LES ring activities were recorded on an online computer using Chart Pro V 4.0 (AD Instruments, New Zealand). The chamber was continuously gassed with 100% O_2 and maintained at 35 °C. The LES ring preparation was gently stretched up to 1 g, and a stable tone was achieved after an equilibration period of approximately 1 h.

2.2. Solutions and drugs

The composition of the normal Tyrode's solution contained (mM) NaCl 136.9, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0, and glucose 5.5 (pH 7.4, adjusted with NaOH). The high- K^+ Tyrode's solution contained (mM) NaCl 92.3, KCl 50, $CaCl_2$ 1.8, $MgCl_2$ 0.5, NaH_2PO_4 0.33, HEPES 5.0, and glucose 5.5 (pH 7.4, adjusted with NaOH).

Carbachol chloride, nicorandil, iberiotoxin (IbTX), nifedipine, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) and sodium nitroprusside (SNP) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Glibenclamide was purchased from Funakoshi Co. (Tokyo, Japan). N^G -nitro-L-arginine methyl ester, hydrochloride (L-NAME), diazoxide, charybdotoxin (ChTX) and pinacidil were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Nicorandil was dissolved in ethanol as a 10 mM stock solution and diluted in the bath solution to give each concentration described in the text. Nifedipine, glibenclamide and pinacidil were dissolved in dimethyl sulfoxide as 10 mM stock solutions. Carbachol, L-NAME, IbTX, ChTX, ODQ, diazoxide and SNP were dissolved in distilled water and diluted in the bath solution.

2.3. Data analysis and statistics

Following incubation in normal Tyrode's solution for more than 1 h, the LES rings were subjected to various drugs, as described in the text. The concentration–response relationship for carbachol was obtained by cumulatively adding carbachol to the organ bath (Fig. 1). The amplitude of the response was measured and normalized with reference to the maximum response. The relationship between the carbachol concentration and the magnitude of the increase in the tension was fitted with Hill's equation,

$$F/F_{max} = 1/(1 + (EC_{50}/[D])^n),$$

where EC_{50} is the half maximal-effective concentration of carbachol and n is the Hill coefficient. In the experiments where the concentration–response relationships for various K_{ATP} channel openers (Fig. 2B) and SNP (Fig. 4) were examined, the magnitude of the inhibition was normalized with reference to the response to 1 μ M carbachol. The relationships were fitted to Hill's equation, except that half-maximal inhibitory concentration (IC_{50}) values were obtained instead of EC_{50} values.

Results are expressed as the mean \pm SEM. Significant differences between drug treatments were determined by analysis of variance, and a P value <0.01 was considered statistically significant.

2.4. Reverse-transcription polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to confirm the expression level of pore-forming and regulatory subunits in the esophageal K_{ATP} channel. Esophageal tissues taken from each part of the esophagus (upper,

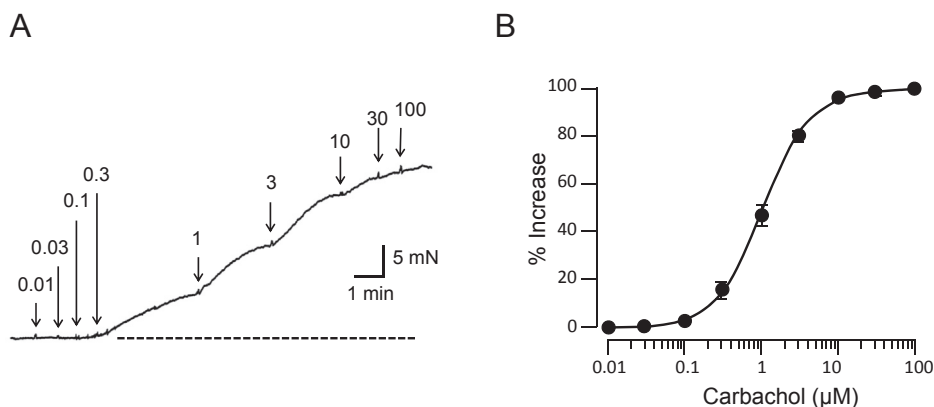


Fig. 1. Concentration-dependent contraction of the rat LES by carbachol. A: A representative recording of the carbachol-contracted LES is shown. Carbachol was cumulatively added to the organ bath so as to obtain the concentration (in μ M) described above the recording. The dashed line indicates the basal tension level. B: Dose–response relationship for carbachol. The smooth curve represents the least squares fit of the data to the Hill equation with a half-saturation concentration of 1.07 μ M. Data are means \pm SEM of 10 experiments.

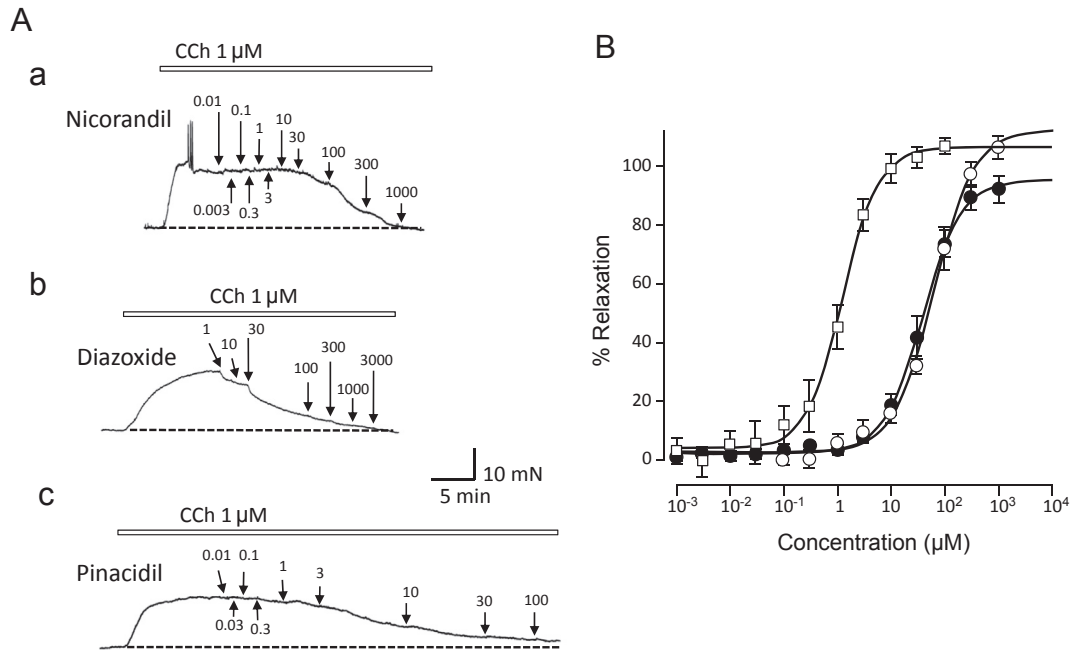


Fig. 2. Inhibition of the carbachol-contracted LES by K_{ATP} channel openers. A: Representative recordings for the effects of K_{ATP} channel openers on the carbachol-contracted LES are shown. After obtaining a stable response to 1 μ M carbachol, nicorandil (a), diazoxide (b) and pinacidil (c) were cumulatively applied to the organ bath at times indicated by the arrow heads. The numbers indicate the concentration of the drug in μ M. The dashed lines indicate the basal tension level. B: Concentration–response relationships for nicorandil (filled circles), diazoxide (open circles) and pinacidil (open squares). Smooth curves were drawn from the least squares fit of the data to the Hill equation with a half-saturation concentration of 37.4 μ M for nicorandil, 83.8 μ M for diazoxide, and 1.25 μ M for pinacidil. Data are means \pm SEM of 10 experiments.

middle and lower) were used as samples. Total RNA was extracted using Isogen II (Nippon Gene, Tokyo, Japan), and 1 μ g RNA was reverse-transcribed using an oligo dT primer in a final volume of 20 μ L. Sequences of primers are as follows: for *KCNJ8* (Kir6.1), forward, 5'-CCGTCTGTGTGACCAATGTC-3', reverse, 5'-CGTGGTTTCTTGACCACCT-3', 350 bp was the size of the PCR product; for *KCNJ11* (Kir6.2), forward, 5'-CCTGGCCATCCTTATCTGA-3', reverse, 5'-TTGGAGTCGATGACGTGGTA-3', 363 bp was the size of the PCR product; for *ABCC8* (SUR1), forward, 5'-CCTGCAGCCAGACATAGACA-3', reverse, 5'-CAGTCAGCATGAGGCAGGTA-3', 276 bp was the size of the PCR product; for *ABCC9* (SUR2), forward 5'-GGAGC-CAAAGCTCAAAGTG-3', reverse, 5'-AGAAGCGCAGGTCTGACATT-3', 354 bp was the size of the PCR product. In the present study, the primer pairs for SUR2 were constructed based on the common regions of both SUR2A and SUR2B, and therefore do not discriminate them.

2.5. Immunohistochemistry

Esophagus including LES was fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned 4 μ m-thick slices longitudinally. Sections were deparaffinized by incubation in xylene (three times by 10 min), followed by washing and rehydration in ethanol (three times by 5 min). Sections were processed by phosphate-buffered saline (PBS) plus 0.1% TritonX100 for increased membrane permeability. The sections were then washed with PBS and blocked with 3% (w/v) bovine serum albumin in PBS at room temperature for 10 min. In order to detect the histological localization of Kir6.1 and SUR2 proteins, the LES sections were next incubated with a goat anti-Kir6.1 primary antibody (NPB1-70370, NOVUS BIOLOGICALS Minneapolis, USA; diluted 1:300) and a rabbit anti-SUR2 primary antibody (LS-C119322, LifeSpan BioSciences, Inc; diluted 1:100) in blocking solution at room temperature for 3 h. Following washing with PBS (3 times for 5 min), sections were incubated with

Alexa Fluor 594 donkey anti-rabbit IgG (A-21207, Invitrogen Carlsbad, CA, USA; diluted 1:100) and Alexa Fluor 488 donkey anti-goat IgG (ab150129, Abcam; diluted 1:200) in blocking solution for 20 min at room temperature in the dark. In order to detect the localization of Kir6.2 and SUR1 proteins, the sections were incubated with a goat anti-Kir6.2 primary antibody (orb19764, Biorbyt Ltd, UK; diluted 1:100) and a rabbit anti-SUR1 primary antibody (orb7031, Biorbyt Ltd, UK; diluted 1:100) in the blocking solution at room temperature for 3 h. Following washing with PBS (3 times for 5 min), sections were incubated with the secondary antibodies. After further rinse with PBS, the sections were incubated for 5 min with DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, Vector Laboratories Inc, Burlingame CA, USA) for nuclear staining. Fluorescence images of stained LES were obtained using a confocal microscope (Zeiss 510, Zeiss, Oberkochen, Germany).

3. Results

3.1. Inhibition of the carbachol-contracted LES by K_{ATP} channel openers

We first examined carbachol concentration-dependent LES contraction. A representative trace is shown in Fig. 1A, where carbachol was cumulatively added to the organ bath, resulting in an increase of LES tension. The carbachol concentration-relationship was obtained for 10 preparations and is illustrated in Fig. 1B. The smooth curve was drawn using the least squares fit to the Hill equation, and the EC_{50} of carbachol was 1.12 ± 0.13 μ M. The Hill coefficient was 1.47 ± 0.02 .

The effects of various K_{ATP} channel openers on the carbachol-contracted LES are illustrated in Fig. 2. After obtaining a stable response to 1 μ M carbachol, K_{ATP} channel openers were cumulatively applied to the organ bath. It was evident that all K_{ATP} channel openers inhibited the carbachol-contracted LES in a concentration-

dependent manner. The concentration–response relationships shown in Fig. 2B revealed IC_{50} values of $41.2 \pm 7.1 \mu\text{M}$ ($n = 4$) for nicorandil, $68.0 \pm 8.8 \mu\text{M}$ ($n = 4$) for diazoxide, and $1.19 \pm 0.18 \mu\text{M}$ ($n = 4$) for pinacidil. The Hill coefficient was 1.42 ± 0.20 for nicorandil, 1.16 ± 0.13 for diazoxide and 1.43 ± 0.44 for pinacidil.

3.2. Possible contribution of K_{ATP} channels and NO to the inhibitory action of nicorandil

The inhibitory effects of nicorandil on the carbachol-contracted LES were examined in the presence of various substances. Glibenclamide, a K_{ATP} channel inhibitor, successfully prevented the inhibitory effect of nicorandil (Fig. 3Aa). On the other hand, L-NAME, an NOS inhibitor or ODQ, a guanylate cyclase inhibitor, did not affect the nicorandil effect (Fig. 3Abc). We also examined a possible contribution of the high-conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) using IbTX. However, IbTX failed to attenuate the inhibitory effect of nicorandil on the carbachol-contracted LES. From 47 preparations, 100 μM nicorandil alone inhibited the carbachol (1 μM)-induced contraction by an average of $59.9 \pm 3.2\%$; this was significantly reduced to $29.9 \pm 2.1\%$ in the presence of 10 μM glibenclamide ($n = 25$, $P < 0.01$). Although the concentration of glibenclamide was increased up 100 μM , no further inhibition was observed ($32.3 \pm 3.9\%$, $n = 19$; $P < 0.01$ vs nicorandil alone). On the other hand, the inhibition was $52.3 \pm 3.2\%$ ($n = 29$), $45.4 \pm 3.6\%$ ($n = 9$), and $56.1 \pm 6.2\%$ ($n = 7$) in the presence of 100 μM L-NAME, 10 μM ODQ and 0.1 μM IbTX, respectively, and no significant difference was observed when compared with the control (100 μM nicorandil alone).

3.3. Inhibition of the carbachol-contracted LES by NO

To examine the effect of NO on the carbachol-contracted LES, SNP, an NO donor, was applied in the presence of 1 μM carbachol. SNP effectively inhibited the carbachol-contracted LES in a concentration-dependent manner (Fig. 4Ba). Fig. 4A shows the

concentration–response relationship for SNP, with an IC_{50} value of $1.86 \pm 0.11 \mu\text{M}$ and a Hill coefficient of 0.93 ± 0.08 ($n = 5$). This inhibitory effect of SNP was attenuated by 10 μM ODQ (Fig. 4Bb). A potential mechanism leading to LES relaxation is NO activation of BK_{Ca} causing membrane hyperpolarization (11–15). However, the inhibitory effect of SNP on the carbachol-contracted LES was not affected by 0.1 μM IbTX (Fig. 4Bc), a highly selective and potent blocker of the BK_{Ca} , or by 0.1 μM ChTX (Fig. 4Bd), a blocker of BK_{Ca} and intermediate conductance Ca^{2+} -activated K^+ channels. On average, 1 μM SNP alone inhibited the carbachol-contracted LES by $76.9 \pm 3.2\%$ ($n = 14$). This was significantly decreased to $21.7 \pm 5.6\%$ ($n = 6$) by 10 μM ODQ ($P < 0.01$). No significant difference was observed depending on whether or not the LES was pre-incubated with 0.1 μM IbTX ($79.6 \pm 4.1\%$, $n = 6$) or 0.1 μM ChTX ($86.3 \pm 2.7\%$, $n = 2$).

3.4. Effect of nicorandil on high K^+ concentration-induced LES contraction

In the experiment shown in Fig. 5A, the LES was contracted by increasing the K^+ concentration of the bath solution from 5.4 to 50 mM and the effect of nicorandil was then examined. Neither nicorandil nor SNP affected high K^+ concentration-induced LES contraction, whereas 1 μM nifedipine significantly attenuated the response. On average, the inhibition was $1.3 \pm 1.9\%$ ($n = 4$) for 100 μM nicorandil, $0.7 \pm 1.8\%$ ($n = 5$) for 10 μM SNP, and $57.1 \pm 4.6\%$, ($n = 4$) for 1 μM nifedipine. These findings are consistent with the view that high K^+ concentration-induced LES contraction is due to the depolarization of LES muscles leading to the activation of L-type Ca^{2+} channels (23). Also, the findings suggest that the inhibitory effects of nicorandil and SNP are caused by K^+ channel activation.

3.5. Expression of pore-forming and regulatory subunits in the LES

Using RT-PCR, the mRNA levels of *KCNJ8* (Kir6.1), *KCNJ11* (Kir6.2), *ABCC8* (SUR1) and *ABCC9* (SUR2B) were determined in the upper,

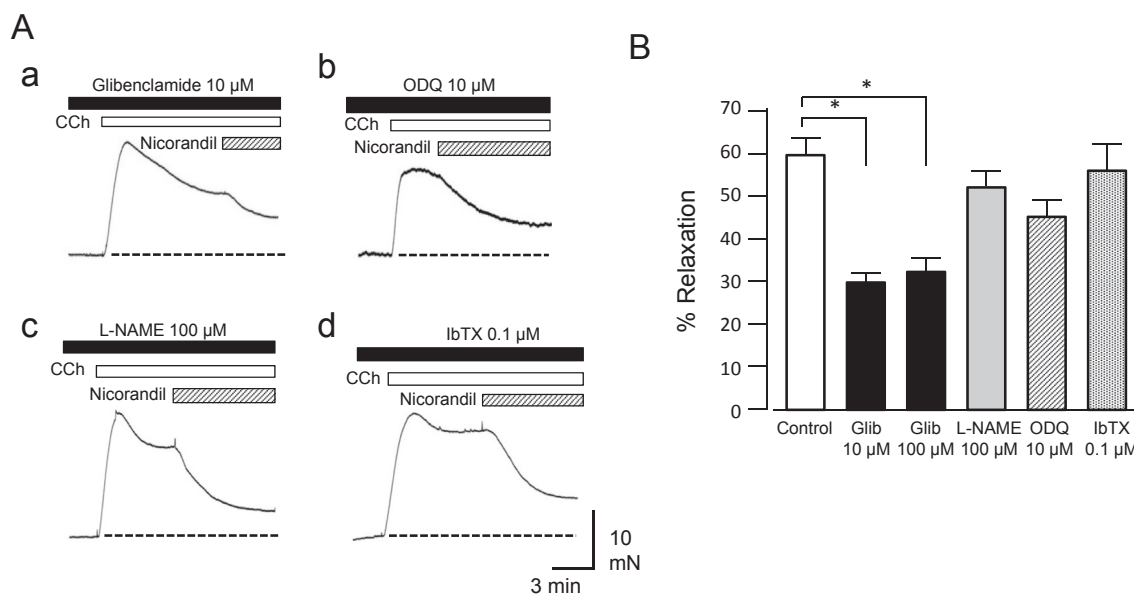


Fig. 3. Inhibitory effects of nicorandil on the carbachol-contracted LES in the presence of various antagonists. A: Representative recordings showing the inhibitory effects of nicorandil on the carbachol-contracted LES in the presence of various blockers. In the presence of 10 μM glibenclamide (a), 10 μM ODQ (b), 100 μM L-NAME (c) and 0.1 μM IbTX (d), the effect of nicorandil on the carbachol-contracted LES was examined using the protocol shown above the recordings. The concentration of nicorandil was 100 μM and that of carbachol 1 μM . The dashed lines indicate the basal tension levels. B: The inhibitory effect of nicorandil was measured in the presence of various blockers. Data are presented as the mean with SEM. * $P < 0.01$ vs control (1 μM carbachol + 100 μM nicorandil).

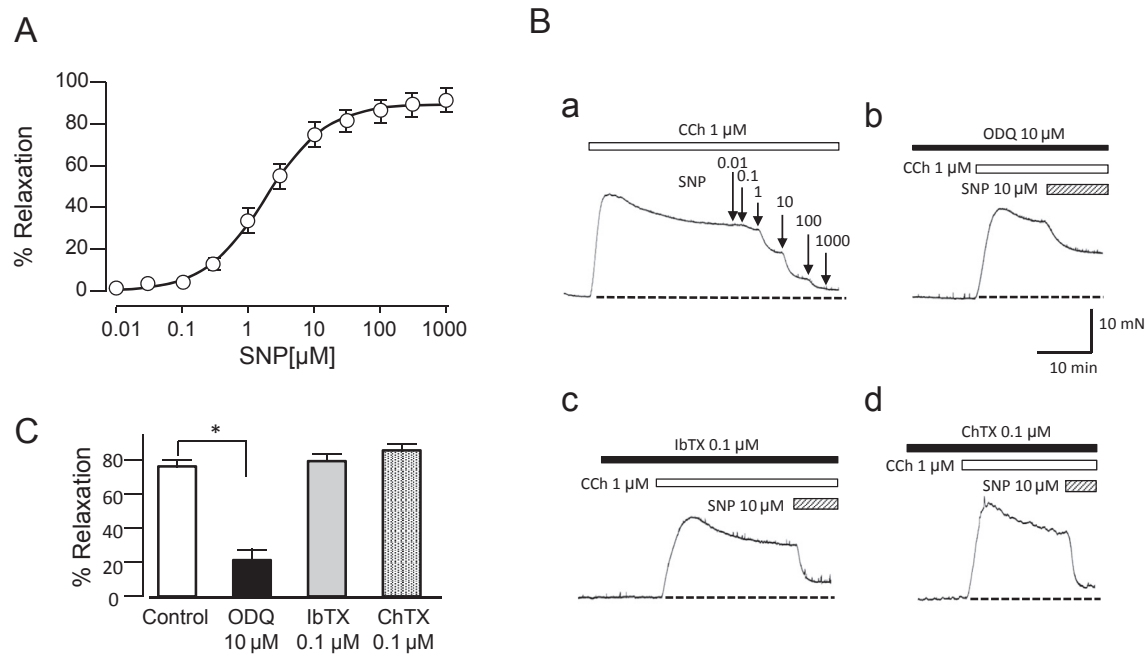


Fig. 4. Inhibitory effects of SNP on carbachol-contracted LES. A: Concentration–response relationship for the inhibition of carbachol-contracted LES by SNP. The smooth curve was drawn from the least squares fit of the data to the Hill equation with a half-saturation concentration of 1.82 μ M. Data are means \pm SEM of five experiments. B: Representative recordings of the inhibitory effects of SNP on carbachol-contracted LES. (a) After obtaining a stable response to 1 μ M carbachol, SNP was cumulatively applied to the organ bath at times indicated by the arrow heads. The numbers indicate the concentrations of SNP in μ M. The inhibitory effects of SNP were examined in the presence of 10 μ M ODQ (b), 0.1 μ M IbTX (c) and 0.1 μ M ChTX (d). The dashed lines indicate the basal tension level. C: The inhibitory effect of SNP was measured in the presence of various blockers. Data are presented as the mean with SEM. * $P < 0.01$ vs control (10 μ M SNP alone).

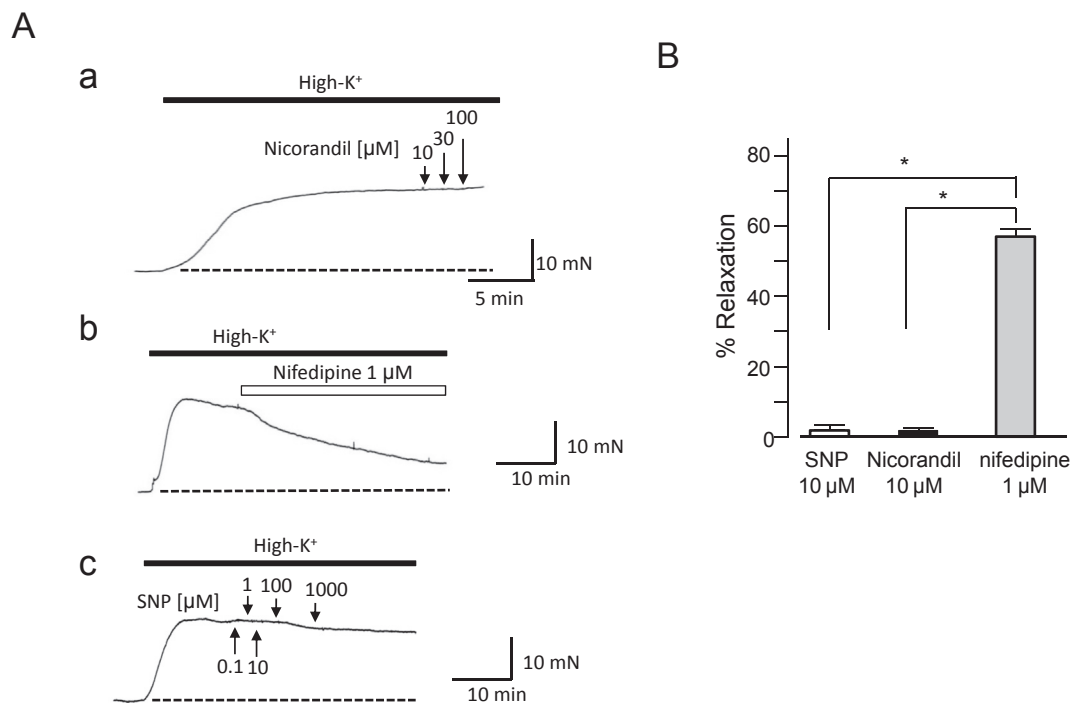


Fig. 5. Nicorandil failed to inhibit high concentration K^+ -induced LES contraction. A: Representative recordings showing the effects of nicorandil on carbachol-contracted LES. After obtaining stable responses to a high concentration K^+ solution, nicorandil (a), 1 μ M nifedipine (b) and SNP (c) were applied as indicated in the recordings. The numbers in (a) and (c) indicate the concentrations of nicorandil and SNP in μ M, respectively. Note that neither nicorandil (a) nor SNP (c) inhibited the high concentration K^+ -induced contraction. The dashed lines indicate the basal tension level. B: Summarized data for SNP ($n = 4$), nicorandil ($n = 5$) and nifedipine ($n = 4$). Data are presented as means with SEM. * $P < 0.01$ vs 1 μ M nifedipine group.

middle and lower (including the LES) parts of the esophagus (Fig. 6). Distinct amplicons of the expected sizes were detected for *KCNJ8* (Kir6.1) and *KCNJ11* (Kir6.2) in all parts of the esophagus. The primer pairs designed to recognize *ABCC8* (SUR1) generated strong bands in the upper and lower parts of the esophagus, and the primer pairs for *ABCC9* (SUR2B) produced relatively weak bands, particularly in the upper part of the esophagus. Similar results were obtained in all PCR assays performed on mRNA extracted from four preparations.

Fig. 7 shows longitudinal sections of the LES. Red and green color in these images represents SURx and Kir6.x, respectively, and DAPI was used for nuclear staining. Both the circular and oblique sling muscle layers were stained with SURx and Kir6.x, and there were no obvious gradient in the expression pattern of circular and longitudinal muscle layers. Similar results were obtained in two other preparations.

4. Discussion

The present study demonstrated that nicorandil inhibited the contractile response to carbachol in rat LES ring preparations in a concentration-dependent manner, and that this effect was prevented, although not completely, by glibenclamide. Inhibition of the carbachol-contracted LES was also observed for other K_{ATP} channel openers, pinacidil and diazoxide. We also showed that SNP, an NO donor, could inhibit the carbachol-contracted LES, an effect that was suppressed by ODQ. On the other hand, neither ODQ nor L-NAME prevented the nicorandil effect. Furthermore, nicorandil failed to affect LES tension when the LES was contracted by a high concentration K^+ -solution. These findings suggest that the relaxing effect of nicorandil on the LES rings is mainly caused by K_{ATP} channel activation, and that the contribution of NO might be relatively small. This is the first study to demonstrate that nicorandil has a relaxing effect on the isolated LES.

K_{ATP} channels are widely distributed among various types of excitable cells, where their primary function is to couple membrane excitability to cellular energy levels (22). K_{ATP} channels in different tissues are made up of different pore-forming and regulatory subunits. In most tissues, *KCNJ11* (Kir6.2) acts as the pore-forming subunit, although in some smooth muscles *KCNJ8* (Kir6.1) serves this role. K_{ATP} channel openers stimulate K_{ATP} channel activity primarily by binding to the regulatory subunit, and native K_{ATP} channels from different tissues display different sensitivities to K_{ATP} channel openers. The differential tissue sensitivity to K_{ATP} channel openers can be attributed to the different types of regulatory

subunits that make up β -cell (SUR1), cardiac (SUR2A) and smooth muscle (SUR2B) K_{ATP} channels (22). Pancreatic β -cell K_{ATP} channels are readily activated by diazoxide, weakly activated by pinacidil, and unaffected by cromakalim or nicorandil. Cardiac K_{ATP} channels are activated by pinacidil, cromakalim, and nicorandil but not by diazoxide. The smooth muscle K_{ATP} channel is activated by all of these drugs (22). In the present study, K_{ATP} channel openers inhibited the carbachol-contracted LES with the potency: pinacidil \gg nicorandil \approx diazoxide. These findings suggest that nicorandil has a relaxing effect on LES smooth muscle by activating K_{ATP} channels that contain SUR2B. Consistently, RT-PCR analysis and immunohistochemistry confirmed the existence of *KCNJ8* (Kir6.1), *KCNJ11* (Kir6.2), *ABCC8* (SUR1) and *ABCC9* (SUR2) in the LES, although discrimination of SUR2A and SUR2B was not performed in the present study.

It has been reported that there is heterogeneity of K_{ATP} expression in a variety of visceral smooth muscle tissues (24). For example, urethral myocytes express the Kir6.2 and SUR1 isoforms in addition to Kir6.1 and SUR2B (25), and heteromeric assembly of Kir6.1 with the Kir6.2 in a (Kir6.1) $_3$ -(Kir6.2) $_1$ arrangement with intermediate channel behavior was suggested (24). Colonic smooth muscle expresses Kir6.2 and SUR2B mRNA with no evidence for Kir6.1 transcript expression (26). Functional characteristics of K_{ATP} channels are also variable, with the predominant nonvascular K_{ATP} subtype recapitulating the salient properties of the K_{ATP} channel, including a low unitary conductance (40 pS), a requirement for nucleoside diphosphates for activation, and similar sensitivity to K_{ATP} channel openers. In this respect, it is interesting to know electrophysiological properties of K_{ATP} channels of the rat LES. Further studies with a help of electrophysiological techniques are clearly necessary to obtain functional evidence of K_{ATP} channels in the LES.

Irrespective of the above heterogeneities, the activation of K_{ATP} channels is expected to drive the membrane potential toward the equilibrium potential for K^+ (19). It has been reported that LES myocytes are more depolarized than those of the esophageal body, displaying a less negative resting membrane potential with relatively high Cl^- conductance (3,27,28). The relatively depolarized resting state of the muscle creates sphincteric tone and favors to the occurrence of spontaneous spike-like action potentials. Cl^- conductance suppression or activation of K^+ conductance may inhibit continuous spike activity and hyperpolarize the cell membrane (28). Thus, activation of K_{ATP} channels would hyperpolarize the cell membrane to prevent Ca^{2+} entry through voltage-dependent Ca^{2+} channels, thereby leading to relaxation of LES myocytes.

Glibenclamide suppressed the 10 μ M nicorandil effect by approximately 30%, and the extent was unchanged even if the concentration of glibenclamide was increased up to 100 μ M. This finding may indicate the existence of mechanisms other than K_{ATP} channel activation. It is generally accepted that NO is the main neurotransmitter for active inhibition and relaxation of the LES (9,29), and the primary mechanism for NO-mediated relaxation is considered to be via a cGMP pathway. In the present study, however, the carbachol-contracted LES was not affected by ODQ or L-NAME. It seems that an NO-dependent mechanism is not involved in the nicorandil effect on the LES contraction.

On the other hand, an externally applied NO donor, SNP, markedly suppressed the carbachol-contracted LES, and this effect was significantly attenuated by ODQ. This finding is consistent with the view that NO is the main neurotransmitter for active inhibition and relaxation of the LES (9,29). There are several potential intracellular pathways whereby NO can lead to smooth muscle relaxation. Involvement of BK_{Ca} has been demonstrated in various species including cat (12), opossum (13), guinea pig (15), dog (10) and mouse (30). The activation of BK_{Ca} causes membrane

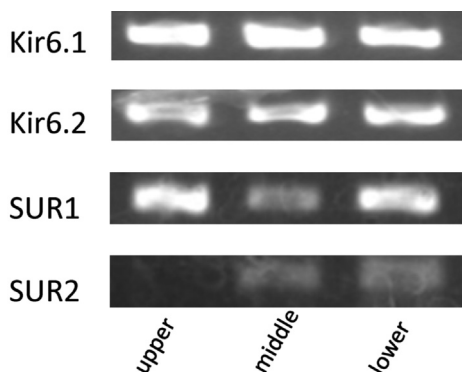


Fig. 6. Expression of pore-forming and regulatory subunits of K_{ATP} channels in the rat esophagus. Comparative reverse-transcription polymerase chain reaction (RT-PCR) analysis of gene expression for *KCNJ11* (Kir6.2), *KCNJ8* (Kir6.1), *ABCC8* (SUR1) and *ABCC9* (SUR2B) in upper esophagus, middle esophagus and LES. Consistent results were obtained for each experiment repeated four or more times.

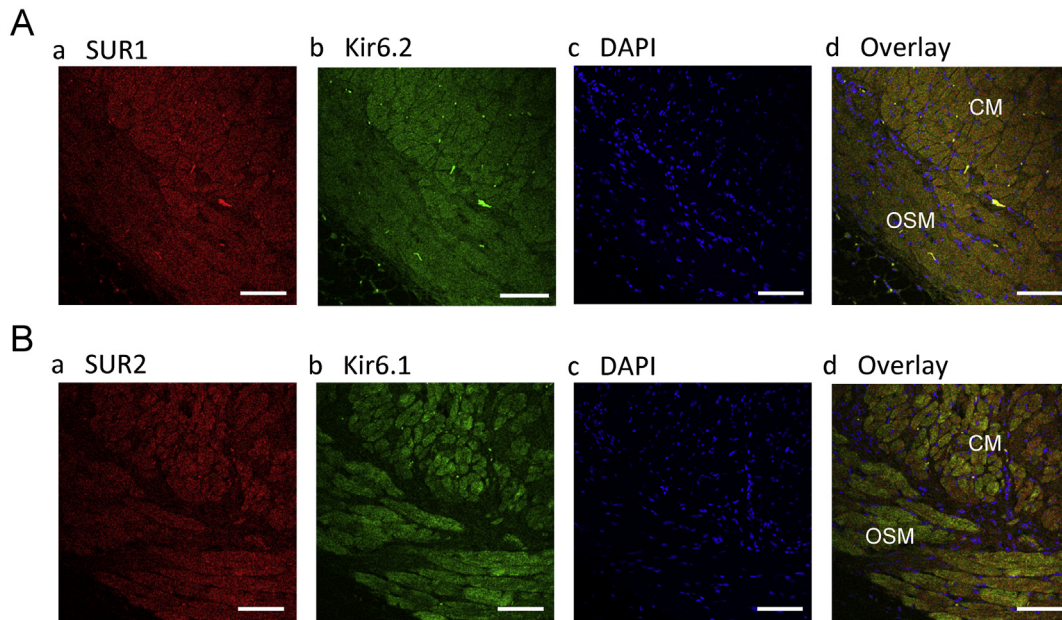


Fig. 7. Images of longitudinal sections of LES showing fluorescent labeling of immunoreactivity for SURx and Kir6.x. A: Immunoreactivity detected with anti-SUR1 antibody (a) and anti-Kir6.2 antibody (b), DAPI nucleic acid stain (c) and an overlay of panels a, b and c (d). Bars (white lines) represent 50 μ m. B: Immunoreactivity detected with anti-SUR2 antibody (a) and anti-Kir6.1 antibody (b), DAPI nucleic acid stain (c) and an overlay of panels a, b and c (d). Bars (white lines) represent 50 μ m.

hyperpolarization (11–15), leading to LES relaxation. In the present study, there were conflicting findings regarding this point. On one hand, blockers of BK_{Ca} , IbTX and ChTX, were ineffective at suppressing the effect of SNP on the carbachol-contracted LES (Fig. 4). On the other hand, SNP failed to attenuate the high K^+ -contracted LES (Fig. 5). The former suggests that BK_{Ca} is unlikely to be involved in the SNP effect, whereas the latter favors K^+ channels mediating the underlying mechanism. We speculate that K^+ channels, which are insensitive to IbTX and ChTX, might have been activated by NO. The responses and the underlying mechanism seem to be different depending on species, the type of muscle, i.e., circular or sling, and on the source of NO. In the canine LES, multiple K^+ channels have been reported to open in response to NO derived from muscle, and NO donors act partly by opening K^+ channels and partly by other mechanisms (11). In the opossum LES, a decrease in Cl^- current and BK_{Ca} activation were observed in response to external application of the NO donor, S-nitroso-L-cysteine (13). Furthermore, L'Heureux et al. reported that exogenous NO-induced relaxation occurred predominantly by a non- BK_{Ca} mechanism in the cat (12); SNP activated the IbTX-sensitive outward current only in circular muscle cells, and SNP reduced tone in both circular and sling muscles, and was unaffected by IbTX (12).

The LES is an important specialized smooth muscle in the gastrointestinal tract, and its dysfunction is thought to underlie various diseases such as achalasia and gastroesophageal reflux disease (5–7). It is well known that non-cardiac chest pain is a common condition defined as recurring, angina-like, retrosternal chest pain of non-cardiac origin. There are many potential etiologies for non-cardiac chest pain, but the esophagus appears to be the most common source for this symptom. The therapeutic options for non-cardiac chest pain patients with esophageal dysmotility, including esophageal spasm, hypercontracting esophagus and hypertensive LES, include medical, endoscopic and surgical treatments. Medical therapy with smooth muscle relaxants, including Ca^{2+} channels blockers, nitrates and phosphodiesterase type 5 inhibitors have shown some level of efficacy in ameliorating symptoms (2,5). The present results suggest that K_{ATP} channel openers,

including nicorandil, may provide an additional pharmacological tool for improving the spastic dysmotility of the esophagus in patients with non-cardiac chest pain. Also, our results might have significant clinical implications for the subset of patients using nicorandil who do not receive full symptomatic alleviation from gastroesophageal reflux disease.

In conclusion, the present study provides evidence that nicorandil inhibits carbachol-induced LES contraction in rats and this was associated with activation of the K_{ATP} channel. Therapeutic strategies that activates K_{ATP} channel may therefore represent an additional option for the treatment of spastic esophageal motility disorders.

Conflicts of interest

None of the authors have any conflicts of interest associated with this study.

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